Pest Management in Horticultural Ecosystems
Vol. 23, No. 1 pp 64-71 (2017)

Induction of systemic resistance in bell pepper (*Capsicum annum* L.) mediated through *Bacillus subtilis* IIHR BS-2 against root-knot nematode, *Meloidogyne incognita*

M. S. RAO, R. UMAMAHESWARI, P. PRABU, M. KAMALNATH, G. N. GRACE and M. K. CHAYA

ICAR-Indian Institute of Horticultural Research, Division of Entomology and Nematology, Bengaluru – 560089, India
E-mail: msrao@iihr.res.in

ABSTRACT: Induction of systemic resistance by *Bacillus subtilis* IIHR Bs-2 established through the activities of defense enzymes *viz.,* catalase, peroxidase (PO), phenylalanine ammonia lyase (PAL) were intensively studied against root knot nematode, *Meloidogyne incognita* in bell pepper (*Capsicum annuum* L. var. *grossum* Sendt) seedlings under *in vivo*. It showed higher accumulation of defense enzymes *viz.,* PAL, catalase and PO during early stages (10-30 days) of bell pepper treated with *B. subtilis* IIHR Bs-2 challenged with *M. incognita* inoculation. The highest enzyme activity was recorded as 136.14, 29.58, 10.72µmol min^{-1} mg^{-1} of protein for PAL, catalase and PO, respectively at 30 days after nematode inoculation. In pot culture experiments, *B. subtilis* IIHR Bs-2 challenged with *M. incognita* inoculation treatment effectively colonized the bell pepper roots and soil (1.8 x 10^4 $cfug^{-1}$ of root and 1.4 x 10^4 cfu g^{1} of soil) and caused significant reduction in nematode population (63.8% in root and 44.14% in soil) together with higher yield (22.05%) compared to untreated control.

Keywords: Biological control, *Capsicum annuum*, induced systemic resistance, *Meloidogyne incognita*

INTRODUCTION

Bell pepper (*Capsicum annuum* L. var. *grossum* Sendt), belonging to family Solanaceae, is used worldwide either as a vegetable or a condiment. Bell pepper is called as capsicum or sweet pepper and also known by other names such as Shimla mirch and green pepper. Bell pepper is cultivated in most parts of the world, especially in temperate regions of Central, South America and Europe, tropical and subtropical regions of Asia mainly in India and China. The mature fruits of bell pepper (green, red and yellow) is gaining popularity in India in the recent years because of its pleasant flavor coupled with rich contents of vitamins especially ascorbic acid and other minerals. India contributes one fourth of world production of capsicum with an average annual production of 18.25 million MT from an area of 2.2 million hectare with a productivity of 8.23 $MTha^{-1}$ (NHB, 2015).

Climatic requirements (tropical and subtropical) for capsicum is however very much conducive for many plant pathogens. Root knot nematode (*Meloidogyne incognita*) is a major biotic constraint hindering successful pepper production in all pepper producing countries. It causes severe root damage that ultimately leads to yield losses to a tune of 12 to 40% (Jain *et al.*, 2007). *M. incognita* parasitizes the roots and changes its anatomy and efficiency by disrupting the vascular system resulting in reduction of water uptake and nutrients transport and ultimately inhibiting photosynthesis (Abad *et al.*, 2003).

Currently, control of *M. incognita* is mainly accomplished through chemical nematicides (Widmer *et al.,* 2000). However, due to the hazardous effects of chemical pesticides on human health and the environment, biological control is emerging as a promising alternative to synthetic nematicides owing to its ability to antagonize the nematodes by different modes of action (Ramamoorthy *et al.*, 2001). The mechanisms of biological control of nematodes by antagonistic bacteria and fungi have been extensively studied for the past two decades (Crickmore *et al.*, 1998). Among these, several plant growth promoting rhizobacteria have been reported to be promising bio-agents with proven excellent characteristics of effective root colonization, versatile activity against multiple nematodes, ability to sporulate and promote plant growth as well (Siddiqui and Mahmood, 1999; Siddiqui and Shaulat, 2003; Radnedge *et al.*, 2003; Kloepper *et al.*, 2004).

Among the rhizobacteria, *Bacillus subtilis* is reported to have direct antagonism towards several genera of

pathogenic nematodes *viz., Heterodera, Meloidogyne* and *Rotylenchulus* (Khan *et al.*, 2008; El-Hadad *et al.*, 2010). The mechanisms involved in biological control affecting root gall development, egg hatching or nematode survival were either directly through the production of toxic metabolites or indirectly by induction of systemic resistance in plants (Siddiqui and Shaulat, 2003; Kloepper *et al.*, 2004; Rao *et al.*, 2009; Abbasi *et al.*, 2014).

The present study aims to investigate the *in vivo* nematicidal potential of *B. subtilis* IIHR Bs-2 against *M. incognita* in order to determine their effect on plant growth and biochemical changes induced indirectly in bell pepper following their application with special focus on three defense related enzyme activities *viz.,* catalase, peroxidase (PO) and phenylalanine ammonia lyase (PAL).

MATERIALS AND METHODS

Bell pepper treatment with *B. subtilis* IIHR Bs-2

The locally isolated and identified *B. subtilis* (IIHR Bs-2; NAIMCC-B-01211) was deposited at NBAIM (National bureau of Agriculturally Important Microorganisms, Mau, Uttar Pradesh, India) and used to study the defense reaction induction in bell pepper (cv. Orobelle; Syngenta) against root-knot nematode. *B. subtilis* IIHR Bs-2 culture was grown in nutrient broth under constant shaking at 150 rpm for 48 h at room temperature (25 ± 2°C). After incubation, the biomass obtained was mixed with pre-sterilized talc powder at 1:3 (v/w) and 2 percent carboxy methyl cellulose as a sticking agent. The talc formulations were dried to 10% moisture content stored at ambient temperature (Rao *et al.*, 2009, 2012). The population of bacteria in the formulation was 2.8 x 10^8cfu/g of talc powder.

Effect of *B. subtilis* IIHR Bs-2 at seedling stage

Bell pepper cv. Orobelle was treated with *B. subtilis* IIHR Bs-2 as seed and substrate treatments. Initially the seeds were treated with bio-agent at 20g/kg and sown in seedling trays containing steam sterilized cocopeat substrate treated with formulation at 10g/kg. Egg masses of root knot nematode, *M. incognita* were collected freshly from the cultures maintained in tomato plants at Nematology glass house, ICAR-Indian Institute of Horticultural Research, Bengaluru. After hatching, the second stage infective juveniles (J_2) were harvested upto 72 h and used for the study. The treatments included T1-*B. subtilis* IIHR Bs-2 treatment without nematode inoculation; T2-*M. incognita* alone; T3-*B. subtilis* IIHR Bs-2 treatment challenge inoculated with nematode and T4-Untreated control plants without *B. subtilis* IIHR Bs-2 treatment and nematode inoculation. Nematodes were inoculated to the rhizosphere at 1 J_2/g of substrate. These experiments were conducted in glass house and seedlings were grown in 50 well protrays. Each treatment was applied to one protray and thus each treatment was given to 50 seedlings in a protray. They were kept in Completely Randomized Design (CRD) and the seedlings were used for further studies.

To evaluate the effect of *B. subtilis* IIHR BS-2 on seedling growth, root colonization and nematode population at seedling stage, ten seedlings from each treatment were uprooted from the protrays after 30 days. Observations were recorded on growth parameters *viz.,* seedling length (cm) and weight (g) and assess root colonization by *B. subtilis* IIHR Bs-2 (cfu g^{-1} root and soil). Root colonization was assessed by following the standard serial dilution technique. Root samples (1g) were washed gently to remove the soil and each root was surface sterilized with 0.1% NaOCl and macerated in pestle and mortar in sterile distilled water. The dilutions were prepared up to 10^{-5} and 0.1 ml aliquots of the 10^{-4} and 10^{-5} dilutions were spread on Petri dishes containing nutrient agar medium and incubated at 27 ± 1°C. The colonies were counted and calibrated to 10^{-4} cfu/ml. After 30 days, seedlings were uprooted and the nematode population in roots g^{-1} was estimated (Bridge *et al.*, 1981). The roots were stained in 0.1% acid fuchsin and nematode population was estimated under binocular stereo zoom microscope (Motic SMZ-168).

Preparation of root samples for enzyme studies

Fresh plant roots (0.5g) were collected at different time intervals (7, 14, 21 and 28 days after nematode inoculation) from the protrays for biochemical analysis and five seedlings were observed for each treatment at each time interval. Fresh roots were washed in running tap water and homogenized using a chilled pestle and mortar and then extracted in 2 ml of 0.05 M Tris – HCl buffer (pH 7.5) including 1mM EDTA and 3 mM $MgCl_2$.The crude extract was centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant was used for assaying of enzyme activities.

Total protein determination

Root extract total protein content was determined spectrophotometrically by recording absorbance at 595 nm (Bradford, 1976). Bovine serum albumin was

used as the standard. Protein content in root samples was recorded as mg of protein per g of root.

Assay of catalase activity (EC 1.11.1.6)

The activity of catalase was measured and the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2 and root extract. The decomposition of H_2O_2 was followed by measuring the decrease in absorbance at 240 nm. The activity was expressed in µmol min^{-1} mg^{-1} of protein (Montavon *et al.*, 2007).

Assay of peroxidase activity (EC 1.11.1.7)

PO activity was performed by measuring the increase in absorbance at 470 nm due to the formation of tetra guaiacol (Sakharov and Aridilla, 1999). The reaction mixture (3 ml final volume) consisted of 2.8 ml 3% guaiacol in 50 mM Tris-HCl (pH 7.0) and 0.1 ml 2% H_2O_2. The reaction was started by adding the 0.1 ml root extract and the absorbance increase at 470 nm was measured. One unit of enzyme activity was defined as the amount of enzyme which produces 1 absorbance change at 470 nm per min in the above assay conditions. The activity was expressed in µmol min^{-1} mg^{-1} of protein.

Assay of phenylalanine ammonia-lyase activity (EC 4.3.1.24)

PAL activity was determined by monitoring the reaction product *trans*-cinnamate at 290 nm (Yan *et al.*, 2008). The reaction mixture contained 50mM Tris-HCl, pH 8.8, 20 mM L-phenylalanine and root extract in a total volume of 3 ml. The reaction was allowed to proceed for 30 min at 30°C and was stopped by the addition of 0.5 ml of 10% trichloroacetic acid. One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 0.01/min under assay conditions. The activity was expressed in µmol min^{-1} mg^{-1} of protein.

Pot culture experiments under protected cultivation

The pot experiment was conducted at Nematology experimental field, ICAR -Indian Institute of Horticultural Research, Bengaluru in pot culture under shade net conditions. After 30 days, the remaining seedlings left out in the protrays (after using in the initial two experiments on seedling growth and enzyme analysis) were transplanted in pots (10 kg) filled with sterilized soil mixtures (3:1:2 of red soil, sand and FYM). For T1 and T3 treatments, *B. subtilis* IIHR Bs-2 was also applied in the pots at the time of planting at 100 g/m^2 after enrichment in vermicompost. For enrichment, two kg of *B. subtilis* IIHR Bs-2 was added to 500 kg of vermicompost and left under shade for 15 days by maintaining optimum moisture (25-28%). Root knot nematodes were inoculated in T2 and T3 treatments after 7 days of transplanting at 1 J_2/g of soil. Each treatment was replicated five times in Completely Randomized Design (CRD). The crop was maintained under shade net conditions by adopting recommended package of practices and the fruits were harvested from 80 days onwards at 10 days interval and yield expressed as kg/plant.

After 150 days, the plants were uprooted and observations were recorded on root gall index on a 1-5 scale (Bridge *et al.*, 1981), nematode population in soil per 100 c.c and root colonization by *B. subtilis* IIHR Bs-2. Nematodes were extracted from soil (100cc) by Cobb's sieving and decanting technique combined with the modified Baermann funnel technique (Cobb, 1918). The nematode population in roots was recorded after staining in 0.1% acid fuschin and observation under stereo zoom microscope (Bridge *et al.*, 1981). Root colonization by *B. subtilis* IIHR Bs-2 was assessed by standard serial dilution technique. All the data were statistically analyzed using SPSS ver. 10.0 software and the analysis of variance (ANOVA) was estimated by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Effect of bioagent at seedling stage

At seedling stage in protrays, *B. subtilis* IIHR BS-2 treated bell pepper recorded significantly higher plant growth parameters. Maximum increase in total plant length (32.89%) and weight (40.15%) over untreated control was recorded in *B. subtilis* IIHR Bs-2 alone treated seedlings, after 30 days. In contrast, root knot nematode inoculated seedlings recorded substantial decrease in total length and weight by 21.46% and 21.25%, respectively compared to untreated control (Table 1; Fig. 2). However, *B. subtilis* IIHR Bs-2 when applied along with root knot nematodes, significantly increased plant total length (28.81%) and (22.04%) weight (Table 1; Fig. 2).Earlier workers have also reported the plant growth promoting potential of *B. subtilis* due to production of indole acetic acid (IAA), siderophore, ammonia and solubilization of inorganic phosphates (Siddiqui 2006; Zaidi *et al.*, 2006; Joseph

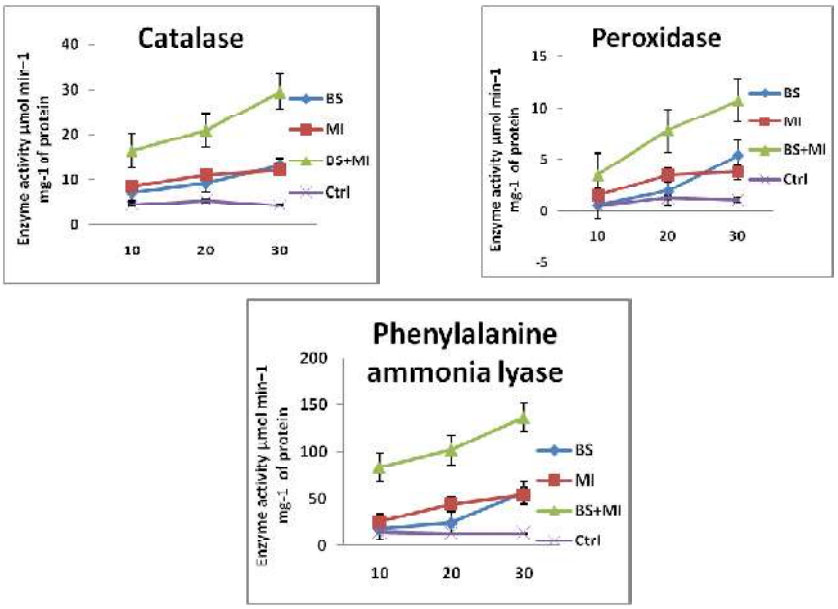

Fig. 1. Expression level of enzyme in bell pepper treated with *B. subtilis* and *M. incognita* : (a). Catalase (b). Peroxidase (c). Phenyalanine ammonia lyase. (BS - *B. subtilis* alone; MI - *M. incognita* alone; BS+MI - *B. subtilis* + *M. incognita*; Ctrl-Untreated control)

et al., 2007). Hence, production of these substances by *B. subtilis* might have contributed to the increase in plant growth parameters reported in the present study.

The root colonization of the bioagent was recorded maximum when treated with *B. subtilis* IIHR Bs-2 alone ($1.8x10^4$ CFU g^{-1} root). It was followed by bacterized plants challenged with root knot nematode where the bacterial colonization was $1.5x10^4$ CFU g^{-1} root (Table 1) and reduction in the nematode population was 61.7% over inoculated control. Similarly Manoj *et al.,* (2013) reported that *B. subtilis* treated bell pepper plant increased the plant growth parameters (shoot and root length) and reduced nematode population.

Enzyme activities

Catalase: In general, there was a higher expression of defense enzymes detected in bacterized plants compared to the non-bacterized plants. After 30 days of

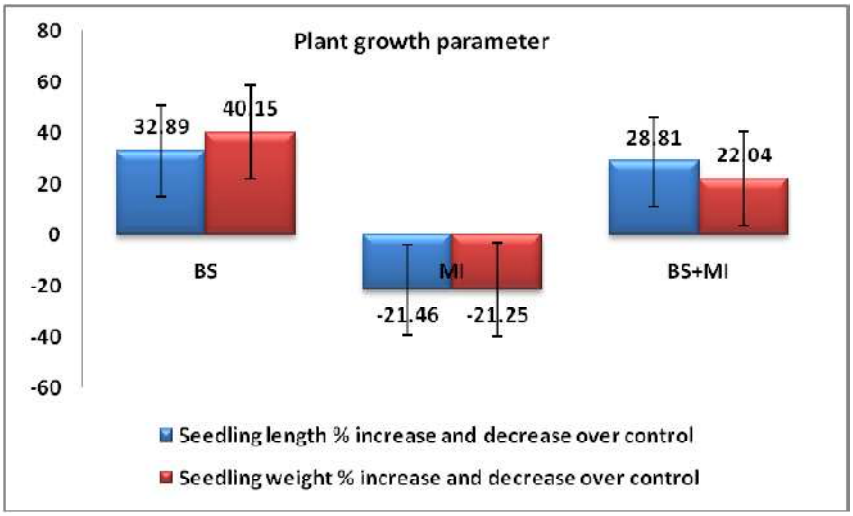

Fig. 2. Effect of *B. subtilis* on the growth of the seedlings of bell pepper (*BS* - B. subtilis *alone; MI* - M. incognita *alone; BS+MI* - B. subtilis + M. incognita)

Table 1. Effect of *B. subtilis* on the growth of bell pepper seedlings and nematode population under glass house conditions after 30 days

Treatment	Seedling length (cm)	Seedling weight(g)	Colonization of *B. subtilis* x10⁴CFU g⁻¹ root	Nematode population g⁻¹ root
B. subtilis alone	14.30	3.56	1.8 (4.25)*	—
M. incognita alone	8.45	2.00	—	45.0 (1.65)*
B. subtilis challenged with *M. incognita*	13.86	3.10	1.5 (4.18)*	17.2 (1.25)*
Untreated control	10.76	2.54	—	—
C.D. at 5%	0.69	0.73	0.01	0.05
SEd	0.32	0.33	0.001	0.02

**Figures in parentheses are log transformed values*

Table 2. Effect of *B. subtilis* colonization on root knot nematode population, gall index and yield of bell pepper after 150 days

Treatment	Nematode population		Root-knot index (1-5)	Colonization of *B. subtilis*		Yield (Kg/plant)
	in 100 cc soil	in 1g root		x10⁵cfu g⁻¹ root	x10⁵cfu g⁻¹ soil	
B. subtilis alone	—	—	—	2.1 (4.26)*	1.5 (4.17)*	18.67 (36.81)#
M. incognita alone	145 (1.95)*	58 (1.76)*	2.32 (0.54)*	—	—	11.69 (-14.32)#
B.subtilis challenged with *M. incognita*	81 (1.90)*	21 (1.31)*	1.24 (0.34)*	1.8 (4.25)*	1.4 (4.16)*	16.65 (22.05)#
Untreated control	—	—	—	—	—	13.64
C.D. at 5%	0.02	0.04	0.05	0.02	0.02	1.16
SEd	0.01	0.02	0.02	0.01	0.01	0.53

**Figures in parentheses are log transformed values*

#Figures in parentheses are % increase/decrease over control

inoculation, the maximum catalase activity was observed in *B. subtilis* IIHR Bs-2 treated plants challenged with root knot nematodes. However there was also increase in catalase activity in *B. subtilis* IIHR Bs-2 alone treated and nematode alone inoculated plants at 10, 20 and 30 days after treatment compared to untreated control. Nematode parasitized plants also exhibited significantly higher catalase activity compared to untreated control (Fig. 1a). Catalase, a stress tolerance enzyme, catalyses conversion of hydrogen peroxide into water and oxygen, which is lethal towards pathogenic microorganisms. Similar trends of enhanced activity of catalase by *B. subtilis* against *Meloidogyne javanica* in brinjal and *Ralstonia solanacearum* in tomato were earlier reported (Li *et al.*, 2008; Abbasi *et al.*, 2014). The present study also indicated that *B. subtilis* induced higher accumulation of catalase upon invasion by *M. incognita.*

PO: Bacterized bell pepper roots upon challenge inoculation with *M. incognita* expressed higher activity of PO compared to either bacteria alone or nematode alone treatments. Maximum PO activity was recorded in plants treated with *B. subtilis* IIHR Bs-2 challenged with root knot nematode after 30 days of inoculation (Fig. 1b). Oxidative enzymes play an important role in plant resistance to biotic stress. Earlier studies reported that *B. subtilis* induced PO activity in response to pathogen *R. solanacearum* attack in tomato (Li *et al.*, 2008; Abbasi *et al.*, 2014). The present study also revealed higher

activity of peroxidase induced in bell pepper by *B. subtilis* IIHR Bs-2 challenged by *M. incognita.*

PAL: The highest PAL activity was recorded in plants treated with *B. subtilis* IIHR Bs-2 along with root knot nematode followed by treatments with *B. subtilis* IIHR Bs-2 alone and nematode alone after 30 days of inoculation compared to untreated control (Fig. 1c).

PAL is the first enzyme involved in the production of phenolics and phytoalexins in phenyl propanoid metabolism against the pathogen establishment (Daayf *et al.*, 1997; Mariutto *et al.*, 2011). The present investigation also revealed increased activity of PAL due to *B. subtilis* IIHR Bs-2 treatment together with nematode parasitization. This falls in line with earlier reports where PAL activity was enhanced by *B. subtilis* in brinjal against *M. javanica* (Abbasi *et al.*, 2014). Also in the present study, increased activity of catalase, PO and PAL was observed in nematode alone treatments, perhaps, due to invasion by the nematode which was also earlier reported against *M. incognita* in tomato (Anita *et al.*, 2004).

Effect of *B. subtilis* IIHR Bs-2 on nematode population and yield

Under pot culture conditions, colonization of *B. subtilis* IIHR Bs-2 was maximum (2.1 10^4 g^{-1} roots and 1.5 x 10^4 g^{-1} soil) when treated alone followed bychallenge inoculation with nematodes (1.8 10^4 g^{-1} roots and 1.4 x 10^4 g^{-1}soil). Significant reduction in gall index (46.6%) and nematode population in roots (63.8%) and soil (44.14%) was recorded in *B. subtilis* IIHR Bs-2 treatment challenged with root knot nematodes (Table 2). *B. subtilis* is reported to produce a broad spectrum of nematicidal volatile compounds (benzene acetaldehyde, 2-nonanone, decanal, 2-undecanone and dimethyl disulphide), antinemic lipopolypeptides and antibiotics (surfactin, fengycin and iturin) which were antagonistic towards *Meloidogyne* spp. (Huang *et al.,* 2010; Kavitha *et al.,* 2012).Thus, in the present study, production of an array of such nematicidal compounds by *B. subtilis* might have caused the reduction in nematode population in roots and soil.

B. subtilis IIHR Bs-2 alone treated plants recorded significantly higher crop yield (36.81%) followed by *B. subtilis* IIHR Bs-2 with root knot nematode inoculation (22.05%) compared to untreated control. However, root knot nematode affected plants recorded 14.32% decrease in yield compared to untreated control (Table 2). These results fall in line with Manoj *et al.* (2013) wherein *B. subtilis* was found effective in reducing root knot nematode population and increasing the bell pepper yield.

Thus the present study proves the induction of systemic resistance in bell pepper plants by *B. subtilis* IIHR BS-2 against *M. incognita* by accumulation of more defense enzymes *viz.*, phenylalanine ammonia lyase, catalase and peroxidase. These bacterized plants also demonstrated increased plant growth and crop yield together with decreased nematode population. This work elucidates the mode of antagonistic action exhibited by *B. subtilis* towards root knot nematode in bell pepper. It is concluded that bacterial strain *B. subtilis* IIHR Bs-2 serves as a vital bio agent and has a great potential as bionematicide against phytonematodes.

ACKNOWLEDGMENTS

The authors thank Dr. G. J. Samathanam, Former Advisor, DST for funding this research work and Director, ICAR-Indian Institute of Horticultural Research, Bengaluru for providing facilities for undertaking this work.

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MS Received : 1 April 2017
MS Accepted : 29 May 2017